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NEW INFORMATION ON THE POTENCY OF SPONGE-ASSOCIATED ACTINOBACTERIA AS PRODUCER OF PLANT GROWTH-PROMOTING BIOACTIVE COMPOUNDS

DWI RETNOWATI, DEDY DURYADI SOLIHIN, MUNIF GHULAMAHDI and YULIN LESTARI*

Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, Indonesia
Tropical Biopharmaca Research Center, Bogor Agricultural University
Jl. Taman Kencana No. 3, Campus IPB Taman Kencana, Bogor 16128, West Java, Indonesia
**E-mail: yulinlestari@gmail.com*

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ABSTRACT

Sponge-associated actinobacteria are known as bioactive compounds producers with various biological functions, but their potency as plant growth promoters is rarely reported. This research aimed to investigate the potency of sponge-associated actinobacteria as plant growth promoters. Sponges used in these study were *Callyspongia* sp., *Callyspongia aerizusa*, *Carteriospongia contorta*, *Chelanoplysilla* sp., and *Diacarnus bismarckensis*. A total of 53 isolates have been isolated from that sponges by serial dilution method. All isolates classified into two groups, including non-*Streptomyces* and *Streptomyces* based on their morphological characters. Screening of sponge-associated actinobacteria isolates showed from 53 isolates there are 47 isolates produced indole acetic acid (IAA), 33 isolates inhibited the growth of *Xanthomonas oryzae*, 29 isolates grew on free N medium, 22 isolates produced HCN, eleven isolates inhibited the growth of *Pyricularia oryzae*, and five isolates had the capacity to solubilize phosphate. The results suggested that sponge-associated actinobacteria have the potency as plant growth promoter candidates and might be as biofertilizer on tidal lands.

Key words: Actinobacteria, plant growth promoters, sponge

INTRODUCTION

Actinobacteria have the ability to produce secondary metabolite with various functional value. Actinobacteria belong to a group of Gram-positive bacteria with high guanine-cytosine content in their DNA and produce mycelium and spores like fungi (Anderson & Wellington, 2001). Various bioactive compounds produced by actinobacteria are beneficial to humans and plants. For the plants, actinobacteria have the ability to produce plant growth-promoting bioactive compounds (Sreevidya *et al.,* 2016).

Actinobacteria as plant growth promoters have direct and indirect mechanisms. The direct mechanisms can be through the production of Indole Acetic Acid (IAA), nitrogen fixation, phosphate solubilization and siderophore. Meanwhile, the indirect mechanisms may be related to HCN production, pathogen growth inhibition, and plant resistance induction (Sathya *et al.,* 2017). Gopalakrishnan *et al.* (2011) reported that five isolates of *Streptomyces* spp. from compost have the ability to increase plant growth by producing extracellular enzymes (proteases, chitinases, cellulases, and lipases), IAA, HCN, siderophores, and biocontrol.

Exploration of plant growth promoting actinobacteria, currently, is still limited on soil and endophyte actinobacteria. Although actinobacteria have been known to be dominant in soils, they have also been found in other habitats, such as freshwater, mangrove litter, seawater, plant tissue and sponge tissue. There has been no published information on the ability of sponge-associated actinobacteria in producing plant growth-promoting bioactive compounds. The aim of this study was to investigate the capability of sponge-associated actinobacteria

* To whom correspondence should be addressed.

to produce plant growth-promoting bioactive compounds.

MATERIALS AND METHODS

Sample collection

Specimens of the marine sponge were collected by scuba diving at a different depths (16, 18, and 21 meters) from Panggang Island, Taman Nasional Kepulauan Seribu, Indonesia. The samples were rinsed with sterile seawater and placed in sterile plastic. All sponges were stored in a freezer before analysis. All sponges were identified by Fisheries Diving Club from Faculty of fisheries and marine science (Bogor Agricultural University) based on morphological characters.

Isolation of sponge-associated actinobacteria

The isolation was done by maceration method using mortar and pestle. The sponge sample was cut into 1 cm^3. The sample was macerated and added with 45 mL of sterilized seawater, then diluted until 10^{-6} with by comparison 1:9 100 µL suspension of two last dilutions was inoculated into the isolation media. Three types of medium, Humic Acid Vitamine Agar (HVA), Starch Casein Agar (SCA) and Malt Extract Agar (MA), were prepared for the isolation of actinobacteria. All medium contained 15 $\mu g.ml^{-1}$ nalidixic acid and 20 $\mu g\ mL^{-1}$ nystatin. The inoculated medium was incubated at 25°C for four to eight weeks.

Morphological characterization of actinobacteria isolates

The actinobacteria colony was characterized based on morphological characteristics including shape, size, elevation, margin, surface, and spore type. The actinobacteria isolates were cultured on Inorganic Salt Starch Agar (ISP4) medium. The spore type of each actinobacteria isolates was observed using a light microscope (Olympus equipped Optilab) with 400× magnification and the other characteristics were observed by using a stereo microscope.

Characterization of plant growth-promoting factors

All isolates were tested for phosphate solubilization on Pikovskaya Agar plate. Isolates were spot inoculated and incubated at room temperature. The size of the halo zone around the colony was measured after seven days of incubation. For the quantitative assay, all isolates were tested on liquid Pikovskaya medium and the pattern of decreasing pH was measured. The soluble phosphate concentration was measured by the stannous chloride method from the supernatant at seven days after inoculation.

IAA production was measured by the colorimetric assay. One agar plug of actinobacteria culture (4 mm in diameter) was transferred into 30 ml ISP2 broth medium containing 200 $\mu g\ mL^{-1}$ L-tryptophan. The inoculated mediums were incubated at room temperature for seven days. A total of 0.5 mL cell-free supernatant was mixed with 1 mL Salkowski's reagent and kept at the darkroom for 30 min until pink color developed. Optical density was measured using spectrophotometer at 535 nm. The concentration of IAA was determined from the standard curve of IAA.

All isolates were tested for nitrogen fixation on a nitrogen-free medium plate. Isolates were spot inoculated and incubated at room temperature for ten days. Nitrogen fixation ability was shown by the growth of colonies. Actinobacteria culture was inoculated into nitrogen-free broth medium and incubated for 14 days at shaker incubator 120 rpm. A total of 1 ml Nessler reagent was added to 1 mL of supernatant and the mixture was added with ammonia-free distilled water up to 10 mL. The optical density was measured using spectrophotometer at 450 nm. The concentration of ammonium was determined from a standard curve of ammonium sulfate ranging from 0.1 to 1 mmol mL^{-1}.

For detecting of HCN production used the ISP2 medium which was amended with 4.4 g glycine L^{-1}. A Whatman filter paper no.1 was soaked in a solution containing 2% sodium carbonate and 0.5% picric acid was put between the base and lid of the petri dish. The plate was sealed with parafilm and incubated at room temperature for seven days. After incubation, the color of the filter paper changed from yellow to orange-brown, indicating the release of cyanide from actinobacteria isolates.

All isolates sponge-associated actinobacteria were screened for their antimicrobial activity against *Xanthomonas oryzae* and *Pycularia oryzae*. A 14 days old ISP4 agar plug of actinobacteria isolate was put on Nutrient Agar (NA) plate overlaid with an overnight NB culture of *X. oryzae* (10^7 colonies forming unit mL^{-1}) and incubated for 24 hr. Antibacterial activity was indicated by the appearance of a halo zone around the actinobacteria colony. Antifungal activity was checked by the dual-culture assay. Antifungal activity was indicated by inhibition zone between *P. oryzae* colony and actinobacteria colony.

RESULTS AND DISCUSSION

Sponge samples

Based on morphological identification (Tropical Pacific Invertebrates 1991), the sponge obtained from the depth of 16 m and 21 m were identified as *Callyspongia* sp. and *C. aerizusa*, respectively. Meanwhile, the sponge samples found at the depth of 18 m were identified as *D. bismarckensis*, *Chelonaplysilla* sp. and *C. contorta* (Table 1).

All sponge samples have specific ability to produce bioactive compounds. *Callyspongia* sp. is known as biologically active natural products because of its ability to produce various bioactive compounds, such as peptides, terpenoids, alkaloids, polyphenols, and sterols. The extract of *C. aerizusa* sponge named Callyaerin G has been known to have anticancer activity in mouse lymphoma cells (L5178Y) and on Hela cells (Ibrahim, 2008). *D. bismarckensis* extract can inhibit *Trypanosoma brucei* which is the causal agent of "sleeping sickness" disease. Gelani and Uy (2016) reported that a 100 µg mL^{-1} of crude extract of *Carteriospongia* sp. can kill 100% of *Artemia salina* L. larvae.

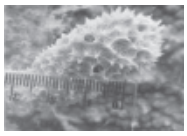
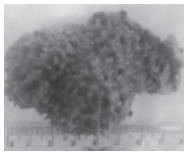
The diversity of sponge-associated actinobacteria

This study showed that the HVA medium was the best medium for actinobacteria isolation, indicated by the most actinobacteria colonies obtained. According to Khannan *et al.* (2011), HVA medium was a selective medium of actinobacteria that can suppress the growth of fast growing bacteria. Simamora *et al.* (2016) also used the HVA medium for isolating 20 actinobacteria from *Neofibularia* sp. sponge.

HVA medium contains humic acid, a complex compound commonly found in the soil and it cannot be used by other bacteria. Actinobacteria can use humic acid as a nutrient source for growth so that it is used as a selective agent in HVA medium.

A total of 53 actinobacteria isolates were obtained from the isolation plates (Table 1). *Callyspongia* sp. exhibited the highest sponge-associated actinobacteria population with a total of 18 actinobacteria isolates. The result indicated that actinobacteria have the ability to associate with all sponge samples. According to Schneemann *et al.* (2010), the presence of sponge-associated actinobacteria contributes to its host defense system, due

Table 1. Sponges-associated actinobacteria

Sponge species	Actinobacteria isolates	Sponges
Callyspongia sp.	Cal1h, Cal2h, Cal3h, Cal4h, Cal5h, Cal6h, Cal7h, Cal8h, Cal9h, Cal10h, Cal11h, Cal12h, Cal13h, Cal14h, Cal15h, Cal16h, Cal1c, Cal2c.	
Callspongia aerizusa	Car1h	
Carteruspongia contorta	Crc1h, Crc2h, Crc3h, Crc4h, Crc5h, Crc6h, Crc7h, Crc8h, Crc9h, Crc10h, Crc11h, Crc12h, Crc13h, Crc14h, Crc15h, Crc16h	
Chelonaplysilla sp.	Che1h, Che2h, Che3h, Che4h, Che5h	
Diacarnus bismarckensis	(Dbi1c, Dbi2c, Dbi3c, Dbi4c, Dbi5c, Dbi1h, Dbi2h, Dbi3h, Dbi4h, Dbi5h, Dbi6h, Dbi7h, Dbi8h	

to the ability of actinobacteria to produce potential secondary metabolite compounds.

Based on the morphological character, especially the spore chains, we found that actinobacteria were divided into two major groups: *Streptomyces* and non-*Streptomyces*. The results showed that 69% of sponge-associated actinobacteria isolates obtained belonged to the *Streptomyces* group with spore chain (Figure 1). According to Anderson and Wellington (2001), *Streptomyces* have spiral spore chains or helix spore chains. *Streptomyces* have branching hyphae to form the vegetative mycelium and they are capable of spreading in the presence of spores.

The ability of sponge-associated actinobacteria to produce plant growth-promoting factors

Screening of sponge-associated actinobacteria isolates showed that 47 isolates produced IAA, 29 isolates can grow on nitrogen-free medium, five isolates have the ability as phosphate solubilization, 22 isolates can produce HCN, 33 isolates can inhibit *X. oryzae* growth and ten isolates can inhibit *P. oryzae* growth (Figure 2). Similarly, John and Thangavel (2015) reported that bacteria isolated from marine sediments are known as IAA producer, nitrogen fixation, phosphate solubilization, and HCN producer.

Phosphate solubilization

Five isolates were able to solubilize phosphate. Based on the qualitative assay, the highest phosphate solubilization activity was shown by Dbi1c isolate with 1.077%. Different from quantitative measurements, the highest phosphate solubilization activity was produced by Cal2c isolate with the soluble inorganic phosphate concentration of 21.14 μg mL^{-1} (Figure 3).

Dastager and Damare (2012) reported that 13 actinobacteria isolate from marine sediments could produce halo zone on Pikovskaya agar medium ranging from 9 to 23 mm after six days incubation and the soluble phosphate concentration ranged from 89.3 to 161 μg mL^{-1}. According to Sing and Dubey (2018), the phosphate solubilization activity is influenced by means of acidification, chelation, redox changes and mineralization of organic phosphorus. In this study, the pH of the medium

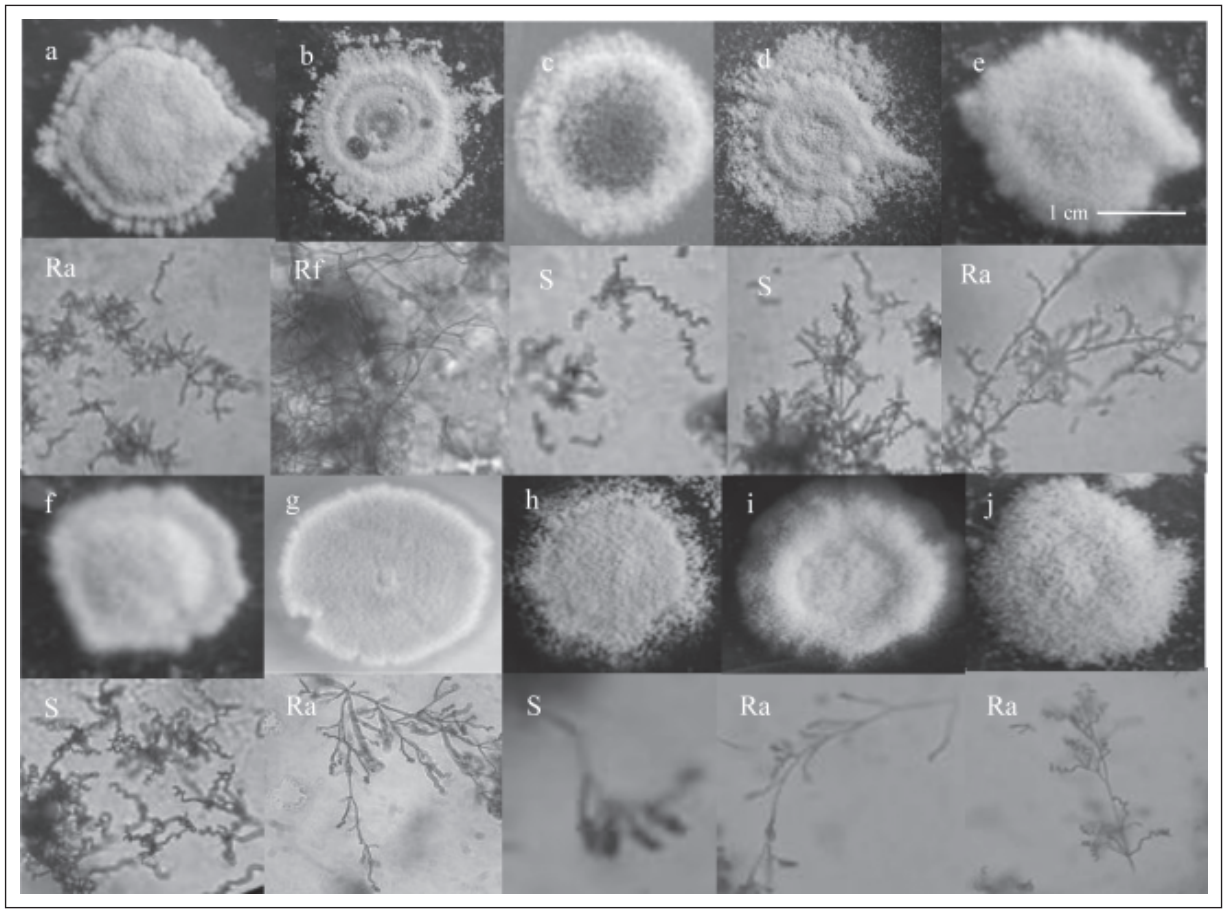


Fig. 1. Morphological colony of sponge-associated actinobacteria after ten days on ISP4 medium (a-j) and spore chain type seen with a 400× magnification light microscope: a. Cal6h, b. Cal1h, c. Cal2h, d. Dbi1c, e. Dbi3c, f. Dbi3h, g. Car1h, h. Crc2h, i. Crc5h, j. Che1h. Spore chain type: Ra= Retinaculiapetri, Rf= Rectiflixibilis, S= Spiral.

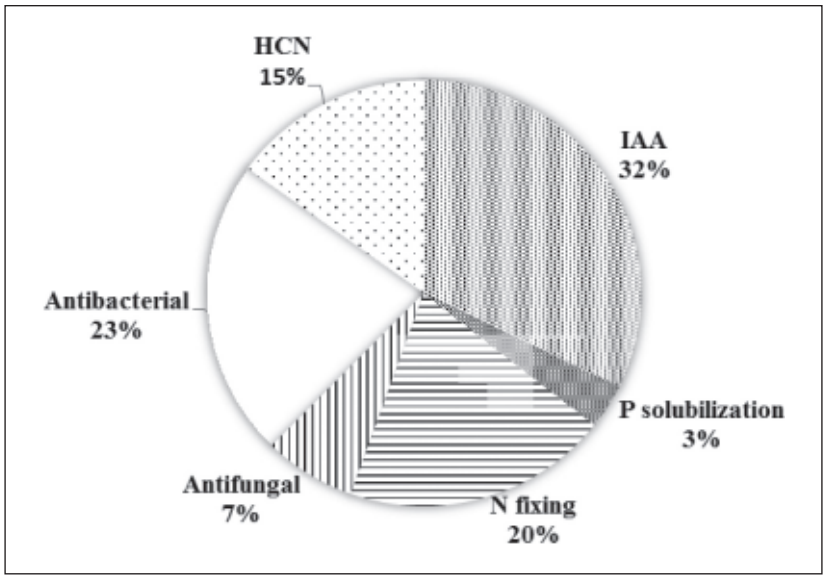


Fig. 2. Capability of sponge-associated actinobacteria in producing plant growth-promoting characters.

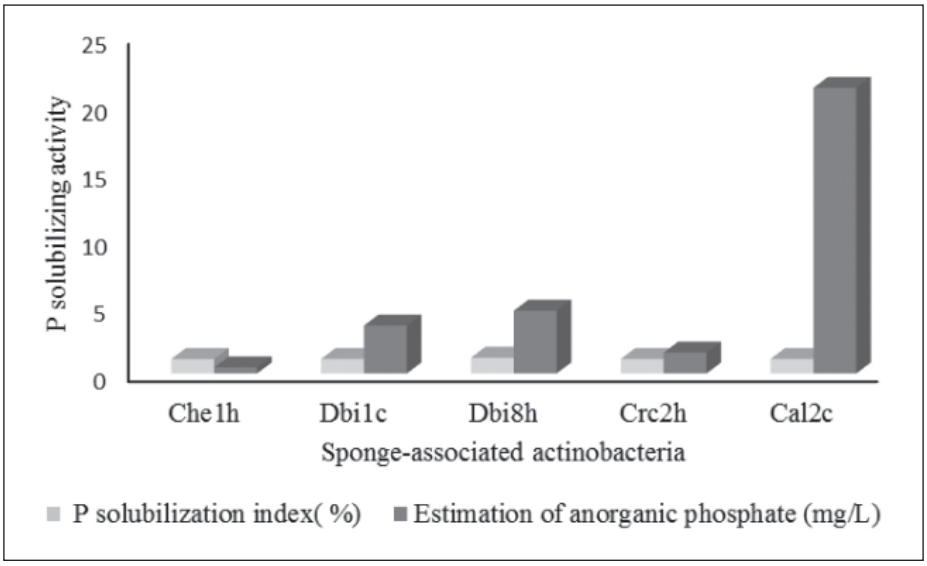


Fig. 3. Activity of sponge-associated actinobacteria in solubilizing phosphate.

decreased from 7 to 5 due to the organic acid compound produced by sponge-associated actinobacteria, such as citrate acid, succinic acid, malic acid, lactic acid, gluconic acid.

IAA production

Sponge-associated actinobacteria have different capacity for producing IAA on liquid medium. The highest IAA production was shown by Crc7h isolate with the IAA concentration of 15.87 µg mL^{-1} after seven days incubation (Table 2). Similarly, Vijayan *et al.* (2012) obtained two actinobacteria isolates from marine sediments capable of producing IAA hormones and found that the highest IAA production was shown by the MB2, with a concentration of 8.6 µg mL^{-1}.

The marine bacteria capable of producing IAA represent promising agents that can be used as biofertilizer in saline fields. Lin and Xu (2013) successfully proved that the IAA biosynthesis pathway of endophytic actinobacteria was also done through the Indole 3-acetamide (IAM) pathway. Trp-2-monooxygenase (IaaM) enzyme will convert tryptophan, the IAA precursor, to IAM and then IAM hydrolase (IaaH) will convert the IAM to IAA.

Table 2. Potency of sponge-associated actinobacteria in produce various plant growth promoting factors

Spnges species	Isolate code	Concentration of IAA (µg mL^{-1})***	N_2 fixation properties		Antifungal against *P. oryzae***
			Growth on N free medium*	Ammonium production (µg.mL^{-1})***	
Callyspongia sp.	Cal1h	5.63	–	0	–
	Cal2h	4.05	+	0.05	–
	Cal3h	8.19	+	0.03	–
	Cal4h	3.15	+	0	–
	Cal5h	3.5	+	0.10	–
	Cal6h	3.74	–	0	–
	Cal7h	0	–	0	–
	Cal8h	0	–	0	–
	Cal9h	10.44	–	0	–
	Cal10h	5.2	–	0	–
	Cal1c	6.25	–	0	–
	Cal2c	5.84	–	0	–
	Cal11h	6.09	–	0	+
	Cal12h	6.19	–	0	–
	Cal13h	1.96	–	0	–
	Cal14h	11.58	+	0.90	+
	Cal15h	7.26	+	0.14	–
	Cal16h	9.54	+	0.013	–
Diacarnus bismarckensis	Dbi1c	4.25	+	0	–
	Dbi2c	3.56	+	0.05	–
	Dbi3c	13.72	+	0.081	–
	Dbi4c	13.68	+	0.014	–
	Dbi5c	0	–	0	–
	Dbi1h	11.34	–	0	–
	Dbi2h	0	–	0	+
	Dbi3h	13.03	+	0.2	–
	Dbi4h	0	–	0	–
	Dbi5h	2.06	+	0.082	–
	Dbi6h	9.82	–	0	+
	Dbi7h	5.84	–	0	–
	Dbi8h	7.69	–	0	–
Callyspongia aerizusa	Car1h	8.01	+	0.01	+
Carteruspongia contorta	Crc1h	4.49	+	0.01	–
	Crc2h	9.32	+	0.08	–
	Crc3h	14.88	–	0	–
	Crc4h	13.06	–	0	+
	Crc5h	14.07	+	0	–
	Crc6h	9.21	+	0	–
	Crc7h	15.87	+	0.15	+
	Crc8h	14,09	+	0,083	–
	Crc9h	14,09	+	0,04	–
	Crc10h	0	–	0	+
	Crc11h	6,15	+	0,83	–
	Crc12h	11,34	+	0	–
	Crc13h	4,62	+	0,031	–
	Crc14h	4,64	–	0	–
	Crc15h	9,09	+	0,048	–
	Crc16h	2,69	–	0	+
Chelonaplysilla sp.	Che1h	6,68	+	0,053	–
	Che2h	5,29	+	0,055	–
	Che3h	4,84	+	0,170	–
	Che4h	3,74	–	0	+
	Che5h	3,05	+	0,053	–

Note:
* : + : able to grow on N free, – : no able to grow on N free medium.
** : + : able to produce antifungal againts, – : no able to produce antifungal againts.
*** : For IAA and ammonium concentration were calculated from duplo measurements.

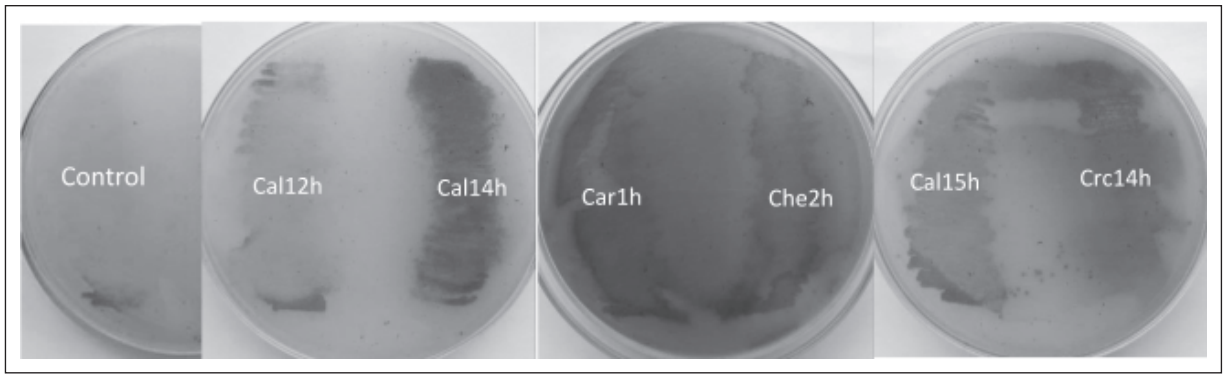


Fig. 4. The HCN production of sponge-associated actinobacteria observed in ISP2 medium containing 4.4 gm glycine L^{-1} after 3 days incubation at room temperature.

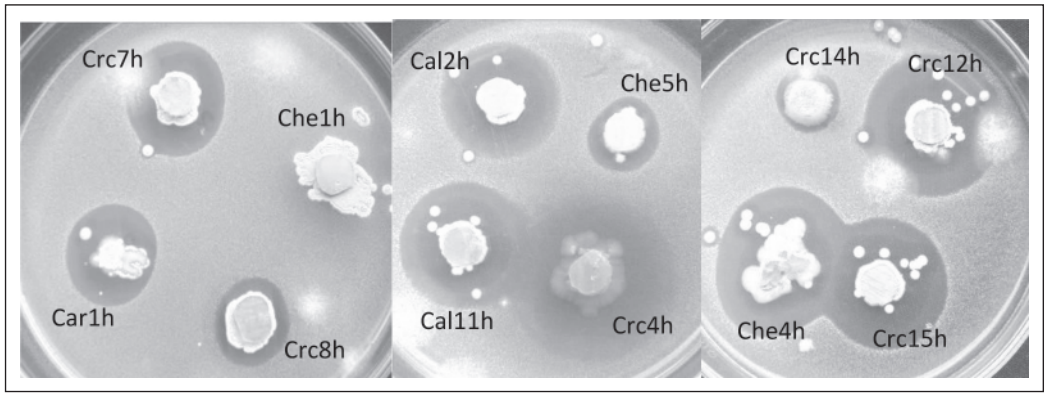


Fig. 5. Inhibition activity against *X. oryzae* by sponge-associated actinobacteria in NA medium after 24 hr.

Nitrogen-fixation

The capability of actinobacteria isolates to grow on an N-free medium indicates that they can fix nitrogen in the air. The result showed that 29 isolates could grow on N-free medium (Table 2). Furthermore, all isolates were used for testing the ammonium production. The highest ammonium concentration produced was 0.83 µg mL^{-1} by Crc11h isolates and the lowest was produced by Crc1h and Car1h isolates of 0.01 µg mL^{-1}. Sari *et al.* (2014) conducted an ammonium production assay to determine the nitrogen-fixing ability of rice endophyte actinobacteria isolates. The results showed that three of seven isolates produce ammonium with concentrations ranging from 0.014 to 0.076 µg mL^{-1}.

Marques *et al.* (2010) recommended that ammonium producing bacteria can supply nitrogen to their host plant. Nitrogen is an essential macromolecule needed by plants, as the constituent of nucleic acid. Plants cannot directly assimilate nitrogen from the air. So the plants need nitrogen-fixing microbes that can provide nitrogen in an available form that plants can absorb.

HCN production

A total of 22 isolates could produce different HCN concentration (Figure 4). This result is in line with Goswami *et al.* (2013), showing that marine bacteria could produce HCN. This isolates had the capability to protect plants from biotic stress, such as the saline environment, via HCN and siderophores production.

HCN is one of the compounds produced by bacteria that play an important role in inhibiting the pathogen growth. Gopalakrishnan *et al.* (2001) reported that actinobacteria endophyte could produce HCN and reduced disease rates caused by *Fusarium oxysporum* by 25%. Sreevidya *et al.* (2016) stated that the actinobacteria capable of producing various plant growth promoting compounds allow balancing the plant rhizosphere. One of them is HCN.

Antibacterial activity

A total of 33 isolates sponge-associated actinobacteria were able to inhibit *X. oryzae* (Figure 5), ranging from 2 to 20 mm. The clear zone was caused by the diffusion of the bactericidal

compound produced by sponge-associated actinobacteria. Antimicrobial compounds produced by actinobacteria may be enzymes or bioactive compounds. The antimicrobial compound can inhibit the growth of other bacteria in three mechanisms: inhibition of bacterial cell-wall synthesis, inhibition of bacterial cell-membrane function, and inhibition of nucleic acid synthesis (Guilhelmelli *et al*., 2013).

In this research, we found that marine actinobacteria have the ability to produce antibacterial bioactive compounds, similar to terrestrial actinobacteria which can inhibit *X. oryzae*. Cheng *et al.* (2015) reported that *Streptomyces* strain MJM4426 isolated from soil could inhibit the growth of *X. oryzae*. The identified compound produced by *Streptomyces* strain MJM4426 is Staurosporine. Staurosporine was first obtained from *S. strausporeus* and *S. roseflavus* (Park *et al.,* 2006).

Antifungal activity

The results of this study showed that ten isolates of sponge-associated actinobacteria could inhibit the growth of *P. oryzae* (Table 2). The results were similar to the research of Awla *et al.* (2016), which successfully obtained *Streptomyces* sp. strain UPMRS4 that can inhibit the growth of *P. oryzae* mycelium with an EIC value 1.562 µg mL^{-1}.

Ten isolates with antifungal activity probably have different inhibitory mechanisms. This study also tested the ability of isolates to produce HCN, six isolates were able to produce HCN. One isolate (Crc10h) could not produce HCN, presumably the inhibitory mechanisms performed by extracellular enzymes production, such as cellulase, chitinase, and glucanase, which can lyse cell-wall of pathogenic fungi. In accordance with the statement of Hamedi and Mohammadipanah (2014) that actinobacteria isolates can combine two different mechanisms to inhibit the growth of pathogenic microbes.

Actinobacteria which have the ability to produce plant growth promoting in order to improve the health of the plants (Sing & Dubey, 2018). Endophytic actinobacteria have several beneficial effects on the host plants, such as inhibition of pathogens, inducing specific genes in the host plant for enhanced disease resistance against phyto-pathogens, and producing phyto-hormones (Ganapathy & Natesan, 2018). It is the first report on the ability of sponge-associated actinobacteria to produce plant growth promoter bioactive compounds. Organisms capable of producing various plant growth promoter bioactive compounds are called as multi-trait Plant Growth Promoting Bacteria (PGPB). The role of actinobacteria as PGPB had been widely reported. Data from this study clearly showed that sponge-associated actinobacteria have the ability to produce IAA, solubilize phosphate, fix nitrogen, produce HCN, inhibit the growth of *P. oryzae* and *X. oryzae*. This phenomenon can be considered as a piece of new information regarding the potency of sponge-associated bacteria having character as a plant growth promoter.

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